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REVIEW

Toxicity assessment of nanomaterials: methods and challenges

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Abstract The increasing use of nanomaterials in consumer and industrial products has aroused global concern regarding their fate in biological systems, resulting in a demand for parallel risk assessment. A number of studies on the effects of nanoparticles in in vitro and in vivo systems have been published. However, there is still a need for further studies that conclusively establish their safety/toxicity, due to the many experimental challenges and issues encountered when assessing the toxicity of nanomaterials. Most of the methods used for toxicity assessment were designed and standardized with chemical toxicology in mind. However, nanoparticles display several unique physicochemical properties that can interfere with or pose challenges to classical toxicity assays. Recently, some new methods and modified versions of pre-existing methods have been developed for assessing the toxicity of nanomaterials. This review is an attempt to highlight some important methods employed in nanomaterial toxicology and to provide a critical analysis of the major issues/challenges faced in this emerging field.

Keywords Nanomaterial toxicology · In vitro · In vivo · Methods · Interference · Challenges

Introduction

The production and use of nanomaterials, which continue to grow, have given rise to many concerns and debates among public, scientific and regulatory authorities regarding their fate in biological systems. Nanoparticles can be classified into two main categories: natural and anthropogenic nanoparticles. Natural nanoparticles existed in the environment long before the nanotechnology era started. Examples of natural nanoparticles include soil colloids, airborne nanocrystals of sea salts, fullerenes, carbon nanotubes, biogenic magnetite, etc. [1, 2]. Soils contain many kinds of inorganic and organic nanoparticles, namely clay minerals, metal oxides and hydroxides, humic substances, allophane, and imogolite [3]. Organic nanoparticles can also be found in natural vegetation [4]. Anthropogenic nanoparticles can be further divided into two categories: incidental, which are nanoparticles produced unintentionally in manmade processes (e.g., carbon black, carbon nanotubes and fullerenes, platinum- and rhodium-containing nanoparticles from combustion byproducts [2]), and engineered/manufactured, which are nanoparticles that are produced intentionally due to their nano-specific properties.

The main focus of current nanomaterial toxicity research is engineered nanoparticles, such as metals, metal oxides, single-walled and multiwalled carbon nanotubes, C-60, polymeric nanoparticles used as drug carriers, and quantum dots. The increase in relative surface area that occurs as particle size decreases down to the nanoscale gives rise to novel and enhanced material properties, but it also renders them more biologically reactive [5, 6]. Reducing particles to nanosize can also give them access to distal regions of biological systems that are normally inaccessible to larger particles [7]. The release of nanoparticles into the environment can occur through many processes, such as spilling and washing consumer products incorporating nanoparticles; during synthesis and production; as an accidental release during transport or use; from industries that exploit nanotechnology, for example wastewater treatment and drug delivery. Environmental contam-

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ination and ecosystem disturbance represent yet another concern. These apprehensions demand the parallel toxicity assessment of nanoparticles alongside their production and application.

Despite the fact that there are a number of publications concerning the undesirable side effects of nanotechnology, the health and safety aspects of nanotechnology have lagged far behind its development. Nanoparticles have been shown to produce cytotoxic, genotoxic, inflammatory and oxidative stress responses in different mammalian cells in vitro [8–14]. The harmful effects of nanoparticles have also been studied in vivo [15–18]. In spite of the presence of voluminous data (Table 1), knowledge about the interactions of nanoparticles with biological systems is still in its infancy. This can be attributed to the many experimental challenges and issues faced when assessing the toxicity of nanomaterials. Most of the methods used for toxicity assessment have been designed and standardized with the chemical toxicity in mind. However, nanoparticles display several unique physicochemical properties that can interfere with or pose challenges to the use of classical toxicity assays. They require much more extensive particle characterization (of factors such as size, shape, solubility, agglomeration, elemental purity, surface area, etc.) than other chemical compounds. Incomplete characterization will hinder attempts to find a correlation between various biological effects and particle properties. Their high adsorption capacities, different optical properties, and increased catalytic activities can influence the results of many in vitro toxicity assays, leading to the misinterpretation of results. Also, an absence of standardized methodologies and guidelines makes it difficult to compare the safety/toxicity assessments from different research groups. This impedes nanotoxicology and results in much apprehension regarding the possible adverse health and environmental implications of nanomaterials.

Several of these methods and the challenges they face from nanoparticles have recently been discussed [19–23]. In this review, we have made an attempt to discuss some important methods employed in the assessment of nanomaterial toxicity, and to perform a critical compilation and analysis of the information available in the literature regarding the main issues/challenges associated with assessing the toxicity of nanomaterials.

Characterization

An initial characterization of the test substance is imperative before any toxicity screening is commenced. However, nanomaterials demand comprehensive characterization, unlike chemical toxicants, where the characterization is usually confined to chemical composition and purity determination. This is because the exact properties of nanoparticles and the reasons for their toxicity are poorly understood. Therefore, a more extensive and complete characterization, including size distribution, shape, surface area, surface chemistry, crystallinity, porosity, agglomeration state, surface charge, solubility, etc., is recommended for nanomaterials in order to determine the correct correlation between their physicochemical properties and the biological effects they elicit. Proper characterization prior to the experiments ensures more repeatability and hence greater reliability of results [24–27]. In addition, the characteristics of commercially available particles that are specified by the manufacturer sometimes differ from those found by the researcher [28]. However, since the facilities in most toxicology laboratories are not fully comprehensive, the complete characterization of nanoparticles is often difficult. In the absence of an elaborate laboratory set-up with all of the instruments and skilled manpower required, researchers are compelled to exploit the techniques available to them. Therefore, sometimes it is the availability of facilities that determines the type of characterization performed rather than the study design or experimental needs.

Among all of the parameters that should be considered for characterization, size is the most important, and it is critical for determining the interactions of nanoparticles with living systems. A variety of methods are available for determining the size of nanoparticles, and the most commonly employed techniques are Brunauer–Emmett–Teller (BET), dynamic light scattering (DLS) and transmission electron microscopy (TEM), scanning electron microscopy (SEM), and atomic force microscopy (AFM) (Table 2). However, another challenge that arises here is the disagreement between average sizes and size distributions given by different methods. This is obviously not surprising in view of the different principles behind the techniques involved. In addition, variations in sample preparation methods and instrument operating procedures also contribute to measurement differences. However, this may lead to confusion about the actual nanoparticle size and size distribution if one is not well versed in the principles and technical details of the measurement methods involved, as is often the case.

The US National Institute of Standards and Technology (NIST) has produced with the world's first reference material (RM) standards of gold nanoparticles for bionanotechnology research. These gold nanoparticles are available in three sizes: 10, 30, and 60 nm. They have been extensively analyzed by NIST for particle size and size distribution by multiple techniques, and details of the measurement procedures used and the data obtained are included in a report accompanying each standard. These RMs are primarily intended for evaluating and qualifying methodology and/or instrument performance related to the

Table 1 Toxicological effects of some widely used nanoparticles (NPs)

Type of NPs	Toxic effects	References
Multiwalled carbon nanotubes (MWCNTs)	In vitro studies: DNA damage, oxidative stress, apoptosis in mammalian cells; they disrupt actin filament integrity and VE-cadherin distribution in human aortic endothelial cells	[81–86]
	In vivo studies: pulmonary toxicity, asbestos-like, length-dependent, pathogenic behavior; induce inflammation, formation of granuloma, cytotoxicity and rapid development of fibrosis in lungs; apoptosis of alveolar macrophages	[87–92]
	Promote allergic response in mice; increased chromosomal aberrations and micronuclei frequency; suppression of systemic immune function through the activation of cyclooxygenase enzymes in the spleen; spotty necrosis, inflammatory cell infiltration into the portal region, hepatocyte mitochondrial swelling and altered gene expression in liver	[93–97]
	Negative effect on reproduction potential, phenotypic defects, apoptosis, delayed hatching and formation of abnormal spinal cords in zebrafish embryo (*Danio rerio*); toxic effects on bacteria	[98–100]
Gold NPs	Affects cellular micromotility; mitochondrial damage; oxidative stress, autophagy in in vitro studies	[101–103]
	In vivo studies: bioaccumulation in important body organs; acute inflammation and apoptosis in the liver; adverse effect on human sperm motility; penetration of gold nanoparticles into sperm head and tail	[104–106]
	Adverse effects on rainbow trout hepatocytes	[107]
Silver NPs	In vitro studies: cytotoxicity and chromosome instability, oxidative stress, apoptosis, intracellular calcium transients, cell cycle arrest, interference with DNA replication fidelity, JNK activation in mammalian cells	[108–114]
	In vivo studies: free radical-induced oxidative stress and alteration of gene expression; blood–brain barrier destruction and astrocyte swelling, neuronal degeneration; induce brain edema formation	[47, 115, 116]
	Cytotoxicity and genotoxicity in fish cells; NPs accumulate in gill tissue; adverse effects on embryonic development of oyster, lysosomal destabilization of adult oysters; oxidative stress, double-strand break marker gamma-H2AX and the expression of p53 protein, embryonic morphological malformations in zebrafish	[117–121]
	Induce heat shock stress, oxidative stress, DNA damage and apoptosis, with upregulation of p53 and p38 proteins in *Drosophila melanogaster*	[122]
	Decrease in reproduction potential, toxicity, oxidative stress in *Caenorhabditis elegans*	[123]
Quantum dots	In vitro studies: cytotoxic, induce inflammatory response, oxidative stress in various types of cell culture systems	[124–130]
	In vivo studies: transfer of quantum dots from pregnant mice to pups across the placental barrier; negative impact of CdSe-core quantum dots on mouse oocyte development; ability to penetrate intact through UV-radiation-compromised skin barrier	[131–133]
	Phototoxic in *Daphnia magna* under environmentally relevant UV-B light	[134]
SWCNT	In vitro studies: impair human macrophage engulfment of apoptotic cell corpses; fibrogenic effects in lung cells; suppress inflammatory mediator responses in human lung epithelium; disrupt actin filament integrity and VE-cadherin distribution in human aortic endothelial cells; activate MAPKs, AP-1, NF-kappaB, and Akt in normal and malignant human mesothelial cells; cause cytotoxicity, oxidative stress, apoptosis, induction of micronuclei and double-strand breaks of DNA; inflammatory response in various mammalian cells.	[82, 86, 135–142]
	In vivo studies: lung inflammation and genotoxicity; increased levels of 8-oxo-dG in liver and lung; activate platelets and accelerate thrombus formation in the microcirculation; promote allergic response in mice	[95, 143–145]
	Microbial inactivation of diverse microbial communities of river water and wastewater effluent; locomotor impairment and mortality in *Drosophila melanogaster*	[146, 147]
Fullerenes	In vitro studies: oxidative stress and DNA-damage potential in different mammalian cells	[13, 38, 148]
	In vivo studies: increase in pro-inflammatory cytokines and Th1 cytokines in BAL fluid, stronger gene expression of the MHC class II molecule than MHC class I and increased T cell distribution in lungs; elevated levels of 8-oxo-dG in the liver and lung	[144, 149]
	Antibacterial activity through reactive oxygen species production	[150, 151]
	Adverse effects on embryonic development in oysters; oxidative stress and growth inhibition in the freshwater fish *Carassius auratus* after chronic exposure; long-term exposure caused significant cellular damage in the alimentary canal of *Daphnia magna*; upon sublethal exposure, the mortality rates of gestating daphnids increased with time and developmental stage, with the maturation of daughter daphnids negatively impacted; extracellular oxyradical and nitric oxide (NO) production, inflammatory response in marine bivalve *Mytilus hemocytes*	[152–156]
Metal oxide NPs	Cytotoxicity, membrane damage, inflammatory response, oxidative stress, apoptosis, lysosomal	[157–170]

Table 1 (continued)

Type of NPs	Toxic effects	References
	membrane destabilization, DNA damage, alteration of calcium homeostasis and gene expression in diverse mammalian systems, as reported in a wide array of in vitro and in vivo toxicity studies; reports of an inhibitory effect of superparamagnetic iron oxide nanoparticles on osteogenic differentiation; disturbance of ionic homeostasis and physiological functions in hippocampal CA3 pyramidal neurons by ZnO NPs	
	Toxic to *Saccharomyces cerevisiae*, bacteria, the nematode *Caenorhabditis elegans*, and aquatic species like fish, phytoplankton, zebrafish	[171–176]

physical/dimensional characterization of nanoscale particles. They may also be useful for the development and evaluation of in vitro assays that are designed to assess biological responses to nanomaterials, and for use in interlaboratory test comparisons.

The nanoparticle surface area is an important factor in nanoparticle toxicity, as the interaction of the nanoparticles with biological systems takes place at their surfaces. The BET method is typically used to calculate the surface areas of solids through the physical adsorption of gas molecules onto the solid surface. It involves adsorbing a liquid nitrogen monolayer onto the surfaces of particles and then measuring the amount of nitrogen released upon vaporizing that layer. Thus, the BET surface represents the surface area that is freely accessible to gases. The primary particle diameter (assumed to be the equivalent sphere diameter) is then calculated from the specific surface area and the density of the particles—data that are already available. Though the merit of this method lies in the fact that it provides two parameters simultaneously (size as well as surface area), it does have a pitfall in that it assumes a monodisperse system of average-sized spheres, so it does not account for the size distribution of the particles, which is a key parameter in size-dependent toxicity assessment [27, 29].

Electron microscopy is the simplest and most widely used technique that directly measures particle size, size distribution and morphology. However, it is time-consuming and requires a sufficient number of particles containing the fields to be analyzed before a sound statistical assessment can be made. Moreover, it measures a sample in dry form, not as a suspension, and requires the drying of samples in vacuum, which may alter their properties. Another drawback of this technique is that it fails to measure the properties of the sample in the form of a dispersion, which is used for experimental exposure [27].

An atomic force microscope (AFM) is a cost-effective instrument that has several advantages in the characterization of nanoparticles. It uses a cantilever with a very thin probe that oscillates over the surface of the sample. An AFM offers visualization in three dimensions with vertical resolutions of less than 0.1 nm and X–Y resolutions of around 1 nm. For individual particles, it provides information on many physical properties: size, morphology, surface texture, and roughness [30]. Unlike other microscopic techniques where the statistics are weak, AFM provides the option of attaining greater statistical significance by carrying out multiple scans. TEM/SEM analysis is generally performed in vacuum, whereas the characterization of nanoparticles by AFM can be performed in ambient air and in liquid dispersions, which may be very advantageous for biological studies. AFM scans also offer a wider range, and particles from 1 nm to 8 μm can be measured in a single scan [31]. Moreover, it requires much less laboratory space than TEM/SEM and is simpler to operate.

Dynamic light scattering (DLS) measures time-dependent fluctuations in scattering intensity produced by particles in Brownian motion, and yields the size of the particle by applying the Stokes–Einstein relation. The size obtained by DLS is usually greater than that measured by other techniques, like TEM, BET, etc. This can be attributed to the fact that DLS measures Brownian motion and the subsequent size distribution of an ensemble of particles in solution and yields the mean hydrodynamic diameter, which is usually larger than the BET or TEM diameter as it includes a few solvent layers [32]. During DLS measurements, there is a tendency of particles to aggregate in the aqueous state, so this method gives the sizes of clustered particles rather than individual particles. DLS reports an intensity weighted average hydrodynamic diameter of a collection of particles, so any sample polydispersity will skew the average diameter towards larger particle sizes [33]. However, the DLS system also affords the option of considering the average hydrodynamic diameter of the particles in terms of number. Considering the particle size in terms of both intensity and number could add value to the analysis.

DLS can measure the hydrodynamic diameter under conditions that more closely resemble the exposure conditions, so it can provide an idea of the particle suspension's stability with respect to time and medium. Murdock et al. showed the utility of DLS by studying the dependence of the in vitro toxicity assessment of nanoparticles on the state of dispersion, the exposure medium, the presence of serum, the time between sample preparation and exposure, etc. [34]. DLS is an ensemble method where the measure-

Table 2 Characterization techniques for nanoparticles

Technique	Parameters analyzed	Comments
Dynamic light scattering	Size, size distribution, agglomeration	• Measures size under conditions that closely resemble exposure conditions • Gives information pertaining to the stability of particles in different media with respect to time • Polydispersity of the sample leads to a bias towards larger particles • Provides average hydrodynamic size, which is usually more than the size measured by other characterization techniques
Nanoparticle tracking and analysis	Size, size distribution, agglomeration	• Allows nanoparticles to be visualized individually with simultaneous analysis of their Brownian motion • Avoids any intensity bias towards large particles
Brunauer–Emmett–Teller	Size, surface area	• Provides two parameters simultaneously: size as well as surface area • Only provides average size, not size distribution
Electron microscopy	Size, size distribution, shape, agglomeration, aspect ratio, elemental composition (when combined with energy-dispersive X ray spectroscopy)	• Direct measurement of particle properties, including chemical composition • Time-consuming • Analyze samples under vacuum • Requires a sufficient number of particles containing the fields to be analyzed before a sound statistical assessment can be made
Atomic force microscopy	Size, size distribution morphology, surface texture and roughness, agglomeration, aspect ratio	• Visualization in three dimensions • Provides information about multiple physical properties • Option of multiple scans for greater statistical significance • Analysis can be performed in the absence of vacuum
Field flow fractionation (FFF; FFF usually combined with other techniques or modified to increase utility: ICPMS–FFF, sedimentation–FFF, flow–FFF [177, 178]	Size, volume, elemental composition (ICPMS-FFF)	• Chromatography-like size-fractionating method that does not utilize a stationary phase • Characterizes particle size via diffusion coefficients
Inductively coupled plasma mass spectrometry [179]	Elemental composition	• High sensitivity • Rapid
Capillary electrophoresis [180]	Size	• Size determination with simultaneous analysis of absorbance properties • Less sample volume required • Rapid • Low operating cost

ment of a collection of particles is used to calculate the particle size distribution.

A more recently developed system based on the Brownian motion of nanoparticles is known as nanoparticle tracking and analysis (NTA). This allows nanoparticles to be visualized individually with simultaneous analysis of their Brownian motion. The particle size distribution can be obtained on a particle-by-particle basis, allowing higher resolution and therefore a better understanding of aggregation than ensemble methods like DLS. It avoids any

intensity bias towards large particles that could result in a small number of large particles/agglomerates masking the presence of a greater number of nanoscale particles, as seen with other light-scattering techniques (e.g., DLS). NTA can be used to identify and count nanoparticle aggregates/agglomerates due to its ability to visualize the particles individually [35]

Analysis of nanoparticle surface composition and structure is generally not given the same importance as size, shape, agglomeration, etc. However, the role of the surface properties of nanoparticles in their toxicity and how these properties are modified during exposure under the influence of different environments needs attention, as they govern the way in which particles interact with biological environments. Electron spectroscopies (Auger electron spectroscopy, AES, and X-ray photoelectron spectroscopy), secondary ion mass spectroscopy, atomic force microscopy, and scanning tunneling microscopy are some surface analytical methods that provide information about topography, elemental composition, molecular and chemical state, and structure [19]. A detailed assessment of all these methods and the technical challenges encountered when applying these surface analysis tools to nanoparticle characterization was made by Baer et al. [19].

In any type of characterization, a consistent powder sampling is the first and most important step. Samples for characterizing nanoparticles and for subsequent toxicity studies are usually taken in small quantities (often mg), but they should be representative of the entire sample. Different ways of performing reliable powder sampling and some common errors associated with sample preparation have already been discussed in detail by Powers et al. [27]. The properties of nanoparticles in liquid suspensions tend to change with time and the surrounding environment. The physical properties of nanoparticles prior to exposure may change once the particles are in the cellular environment, again placing the emphasis on characterization at different experimental steps.

Although the choice of a particular characterization technique depends on the type of particle being analyzed and the final application of the nanoparticles, it is advisable to perform multi-technique analysis in order to get a broader perspective and a more reliable picture of the particle characteristics. Collaboration between different laboratories that possess expertise in their respective techniques needs to be encouraged. A sufficient number of nanoparticles should be measured to get statistical accuracy.

Nanoparticle internalization in biological systems

Tracking nanoparticle internalization in cellular systems is of the utmost importance for understanding and correlating the biological effects elicited by these nanoparticles. However, the challenge lies in detecting the uptake of nanoparticles, the mode of uptake, and the fate of nanoparticles inside the cells due to their small size and quantity.

Transmission electron microscopy has been the preferred method of studying the cellular uptake of nanoparticles. Apart from detecting the intracellular localization, it provides a detailed view of the interaction of nanoparticles with cell structures. Due to its high resolution, transmission electron microscopy enables the imaging of membrane invaginations, vesicle formation, and organelles [36]. This makes it possible to study the mode of nanoparticle uptake, which is of primary importance for understanding the influence of size, shape, surface chemistries, coatings, and other factors on nanoparticle uptake [37, 38]. It also aids in understanding the ultrastructural changes that occur in cells subsequent to nanoparticle uptake [17, 39]. However, transmission electron microscopy is only a qualitative tool for assessing nanoparticle uptake, and is usually confined to imaging a few cells due to the complicated sample preparation and image analysis involved.

A scanning electron microscope (SEM) can also be used to observe nanoparticles inside cells. For this, backscattered electron detection is used instead of the normal secondary electron mode of detection. Backscattered electrons (BSE) are high-energy electrons that are reflected or backscattered out of the specimen following elastic scattering interactions with specimen atoms [40, 41]. This enables bright nanoparticles to be seen against the cellular dark background, since high atomic number elements backscatter electrons more strongly than low atomic number elements. In addition to visualizing the specimen, elemental analysis of the sample can be achieved by energy-dispersive X-ray spectroscopy (EDS) [42, 43]. The staining procedures generally used for electron microscopic preparations can introduce electron-dense artefacts that may be mistaken for nanoparticles [44]. Therefore, SEM-EDS provides more detailed confirmatory evidence on nanoparticle uptake.

Advances in transmission electron microscopy are now offering additional advantages for nanoparticle uptake assessment. The heavy metal stains that are used to increase contrast in TEM can also obscure differentiation between carbon nanomaterials and carbon-rich cellular components due to similarities in composition and dimensions. Energy-filtered transmission electron microscopy (EFTEM) in conjunction with electron energy loss spectroscopy (EELS) have been employed to overcome this challenge [45]. In EFTEM, only electrons with particular kinetic energies are used to form the image. Electrons undergoing inelastic scattering lose some energy, which can be measured by an electron spectrometer. By utilizing electrons with a well-defined energy loss (ionization edge), elemental distribution maps can be generated [46]. In their study, Porter et al. achieved improved contrast between single-walled carbon nanotubes

(SWCNTs) and cell organelles without staining by employing EFTEM [45].

Sometimes very small amounts of nanoparticles in the environment or in living systems make it difficult to perform a qualitative assessment by microscopic tools. Moreover, electron microscopic techniques become ineffective when it comes to the analytical quantification of nanoparticles. In this case, inductively coupled plasma mass spectroscopy (ICP-MS) can be used as a sensitive and quantitative tool for the determination of even trace amounts of nanoparticles. ICP-MS becomes especially important in the context of an in vivo scenario, where it identifies the target organs for nanoparticles [47]. Using this technique, even trace amounts of nanoparticles that enter through a different route can be detected in various body organs. However, the digestion step involved in the sample preparation method for ICP-MS may lead to contamination and dilution, and it makes it difficult to differentiate between ions formed as a result of nanoparticle dissolution and nanoparticles per se [44, 48].

Flow cytometry is yet another technique that can be used to study nanoparticle uptake in mammalian cells [49–52]. It is not only simple, easy and sensitive, but it is also a cost-effective and noninvasive approach. In this method, a laser beam is made to strike a hydrodynamically focused stream of fluid containing a single cell suspension, and a number of detectors then collect information on how the light interacts with the cells. Some of the photons that hit the edge of the cell are deflected slightly, and this forward-scattered light corresponds to the size of the cells. Photons scattered at right angle to the laser beam (side scatter) indicate the inner complexity or granularity of the cells. Fluorescence emitted by the structures present inside the cells or attached to the cells is also picked up by the detector, providing an array of useful information (Fig. 1). Thus, flow cytometry can be used for the detection of fluorescent as well as nonfluorescent nanoparticles inside cells. In the case of the cellular uptake of nonfluorescent nanoparticles, the forward-scattered light remains constant in exposed and unexposed cells, while the intensity of side-scattered light increases in proportion to the concentration of nanoparticles inside the cells. The sensitivity of side scattering should be kept low in order to detect a broad range of changes in uptake [50]. Sample preparation for flow cytometry analysis is much simpler than for other analytical techniques. Cells exposed to nanoparticles are washed, trypsinized and then resuspended in buffer for flow cytometry acquisition. Suzuki et al. used flow cytometry to show that the TiO_2 nanoparticles were taken up by the cultured mammalian cells in a dose-, time- and size-dependent manner [50]. In addition, they also revealed a change in the uptake potential on a surface coating, which was shown by the intensity of the side-scattered light [50]. The uptake of fluorescent amphiphilic hydrogel nanoparticles by a murine macrophage cell line (J774A.1) was demonstrated by determining the fluorescent intensities of exposed cells [49]. The uptake mechanism was also elucidated by selectively inhibiting cellular internalization processes with a variety of inhibitors and then analyzing cells by flow cytometry [49]. The applicability of flow cytometry for studying the cellular internalization of nanoparticles was also utilized in an in vivo study investigating the phagocytic uptake of nanoparticles by mouse peritoneal macrophages [51]. Flow cytometric analysis of nanoparticle uptake in cells can be further supported by fluorescent spectroscopy or microscopy data in the case of fluorescent nanoparticles. Despite its many advantages, the main drawback of flow cytometry in nanoparticle uptake studies is that it can only show the association of nanoparticles with cells; it cannot indicate their localization and fate inside the cells.

Interference of nanoparticles with in vitro toxicity assays

In vitro experimentation has always been the first choice for toxicologists, since it is time- and cost-effective. Although

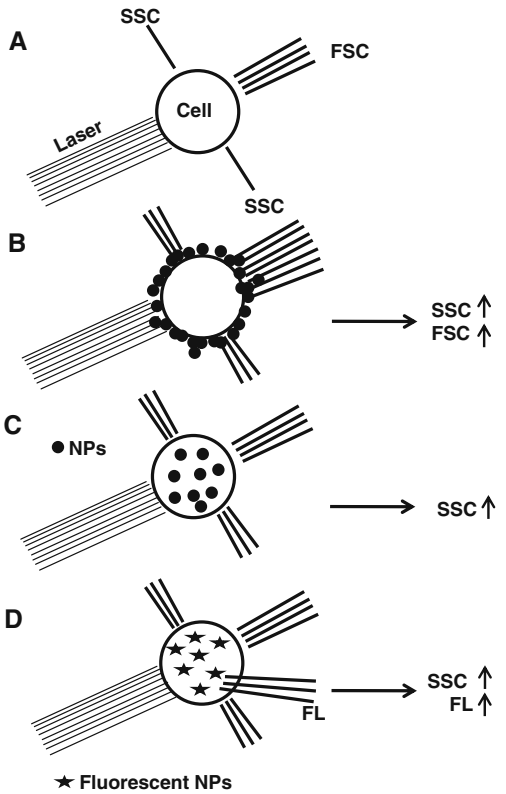

Fig. 1 **A**–**D** Analyzing nanoparticle (NP) uptake in cells by flow cytometry: **A** light scattering by a cell that is not associated with any nanoparticle; **B** nanoparticles adhere to the cell surface, leading to an increase in forward scatter (FSC) and side scatter (SSC); **C** nanoparticle internalization by the cell, leading to an increase in SSC alone; **D** fluorescent nanoparticle internalization by the cell, leading to an increase in SSC and fluorescence intensity (FL)

it cannot replace animal experimentation completely, but it does help to ensure that they are only used when absolutely necessary, and it sometimes provides mechanistic information on the toxicity of nanoparticles after in vivo studies.

The risk assessment of different aspects of nanotechnology is still in its early stages. Therefore, most of the studies pertaining to nanoparticle toxicology that have been carried out so far have been preliminary and confined to the classical in vitro toxicity test methods established for drugs and chemicals. However, the methods that are used in traditional toxicology cannot be applied per se to nanoparticle toxicology, as nanoparticles display several unique physicochemical properties. Due to these properties, nanoparticles interfere with normal test systems, and this interference has been well documented in the literature [20, 39, 53–56]. Examples of such properties include: high surface area, leading to increased adsorption capacity; different optical properties that interfere with fluorescence or visible light absorption detection systems; increased catalytic activity due to enhanced surface energy; and magnetic properties that make them redox active and thus interfere with methods based on redox reactions (Fig. 2) [53]. These obstacles lead to conflicting reports and the generation of unreliable data [20, 55, 57].

Single-walled carbon nanotubes (SWCNTs) interact with a variety of indicator dyes employed in commonly used cytotoxicity assays, such as 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt (WST-1), Coomassie blue, alamarBlue, and neutral red. The nanotubes bind formazan crystals and stabilize their chemical structure, meaning that these crystals cannot be solubilized. These crystals can be found with or attached to carbon nanotubes. This interference of carbon nanotubes with the MTT assay can be attributed to the unusual rope-like structure of this class of nanomaterials compared to other nanoparticles [57]. However, Monteiro-Riviere et al. suggested that SWCNTs and carbon black alone (in the absence of cells) can interact with the dye to cleave the tetrazolium ring and cause a false-positive reaction [55]. This interference with the MTT assay is not confined to SWCNTs alone; it has also been reported for carbon black nanoparticles, silica, and ultrafine superparamagnetic iron oxide nanoparticles [20]. The high adsorptive capacities of nanomaterials have also been reported to interfere with annexin V/PI binding and ELISA tests for cytokine responses [54, 58]. Aam and Fonnum noted fluorescence quenching by carbon nanoparticles while detecting reactive oxygen species generation via dichlorofluorescein (DCF) [59]. Doak et al. demonstrated in a cell-free system that dextran-coated iron oxide nanoparticles interfere with the fluorescence emission of DCF, depending on the concentration of the dye and the oxidation state of iron [20]. They suggested that adsorption could be a reason for the quenching of the fluorescence response [20]. The optical properties of nanoparticles may also influence the results of absorbance-based detection systems, as reported in the case of sodium titanate nanoparticles [60].

The biological effects exhibited by nanoparticles can be associated indirectly with contaminants introduced during manufacturing or while handling them in the laboratory. Moreover, as they are prepared in unsterilized environments, they may harbor some endotoxins too. Although particle purity is stringently checked to avoid these kinds of contaminations, it is not possible to completely rule out any possibility of the presence of contaminants leading to toxicity. Pulskamp et al. demonstrated a dose- and time-dependent increase in intracellular reactive oxygen species with commercial SWCNTs in the rat alveolar macrophage cell line (NR8383) and the human alveolar epithelial cell line (A549), whereas incubation with purified SWCNTs had no effect [56]. They concluded that metal traces that are used during the production process of the carbon nanotubes (CNTs) remain associated with them and are responsible for the biological effects shown by the CNTs [56].

It is important to carefully analyze the interactions of nanomaterials with various components of the toxicity assay before the start of the study. Diverse imaging techniques like TEM can help to highlight any interference or direct interaction with the assay components. Moreover, it is always beneficial to assess safety/toxicity with two or more independent test systems to validate the findings.

The clonogenic assay or the colony formation assay is an in vitro cell survival assay based on the ability of a single cell to grow into a colony. It is a simple method that can be employed to avoid interference from nanoparticles, as no dye or stain is used [61]. However, the complete removal of nanoparticles during the washing step is uncertain.

The various types and degrees of contamination (metal contents and bacterial endotoxins) introduced into the nanomaterials during the production process or post-production handling should be checked for despite the manufacturer's claim of no contamination. Therefore, chemical characterization should accompany the physical characterization. There should be a standardized nanoparticle reference material that can be used by all toxicologists, so that data can be compared across different studies.

Agglomeration and dispersion

The phenomenon of agglomeration involves the adhesion of particles to each other, mainly because of van der Waal's forces, which dominate at the nanoscale due to the increased surface area to volume ratio [27]. It is well known that the nanoparticles start to agglomerate after their

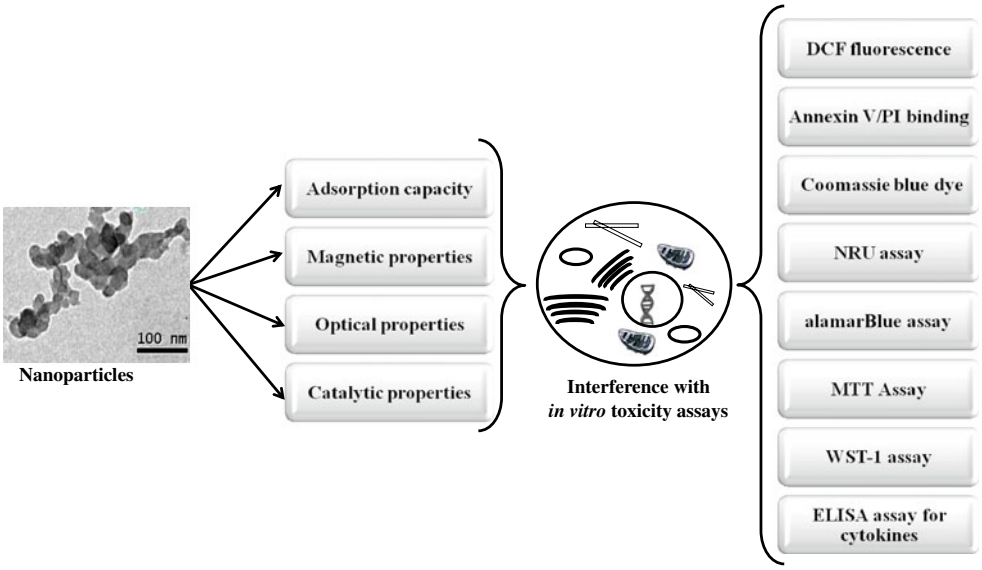

Fig. 2 Nanospecific properties leading to interference with some commonly used in vitro assays

synthesis, both in the dry form as well as in suspension. The challenge for synthetic chemists is to prevent nucleation to ensure that the nanoparticles do not agglomerate, especially in biologically relevant fluids. Brownian motion, in combination with van der Waal's forces, also contributes significantly to the agglomeration of nanoparticles, which ultimately settle down due to gravitational forces. Due to agglomeration, the physicochemical properties and the number concentration of the nanoparticles get altered. The major properties affected are their size, size distribution, surface-to-volume ratio, and hence their surface reactivity. Since these parameters play a major role in the toxicity of nanoparticles, and are altered due to agglomeration, it is prudent to account for these changes in the study design [62, 63].

Agglomeration is influenced by several intrinsic and extrinsic factors, such as the composition of the nanoparticle and its concentration, size, surface coating, dispersant characteristics (pH, presence of serum, salt and surfactant), zeta potential, sonication time, temperature, etc. [63]. It has been shown that the nanoparticle size varies in different dispersion mediums like deionized water and cell culture media with and without serum. Murdock et al. demonstrated that agglomeration decreases in the presence of serum since the proteins coat the particles, providing them with steric stabilization and thereby reducing agglomeration [34]. For instance, when introduced into deionized water and RPMI-1640 media, copper (40 nm) agglomerated with a 28-fold increase in size, but only a ninefold increase compared to its primary size was observed when it was put into media with serum [34]. Therefore, agglomeration is a challenge to the accurate interpretion of the biological response to any given nanomaterial. This is evidenced by an increase in the cytotoxicity when mesothelioma cells (MSTO-211H) are exposed to micron-sized agglomerates of carbon nanotubes rather than well-dispersed carbon nanotubes (dispersed with a nonionic biocompatible surfactant, PS80). This is because the structural characteristics of the material change after agglomeration; it becomes stiffer and behaves like asbestos particles [64].

It is well known that nanoparticles can traverse through biological barriers due to their size. Hence, agglomeration could alter biological responses due to a decrease in the total available surface area, leading to an underestimation of toxic potential, especially in the case of drug delivery and safety/toxicity assessment [65]. The rate and extent of agglomeration of nanoparticles could vary after they enter cells, due to their interaction with macromolecules [66].

Though different methods are available to deagglomerate nanoparticles (sonication, detergents, lung surfactants, polyethylene glycol, serum, etc.), sonication is the most preferable and widely used method. It disperses nanoparticles in a liquid by cavitation and does not have much effect on the properties of the particles. However, the deagglomeration attained is not complete (i.e., particles do not reach their primary particle size and show a tendency to reagglomerate over time [34]). The effect of probe sonication on the agglomeration and surface charge was evaluated by Murdock et al. [34]. Using the size distribution and zeta potential, they demonstrated that probe sonication for different time periods does not disperse the particles to their primary particle size; nor does it provide a lasting stable suspension of nanoparticles [34].

Another important method of preventing the agglomeration of nanoparticles and homogeneously dispersing them in liquids is surface modification. This can be achieved in various ways, depending on the application. The particles can be coated with polymers or dispersed in ionic or nonionic surfactants or alveolar surfactants [64, 65, 67, 68]. Sager et al. compared the dispersion capabilities of various

suspension media: phosphate-buffered saline, rat and mouse bronchoalveolar lavage fluid (BALF), and PBS containing dipalmitoyl phosphatidylcholine (DPPC), mouse serum albumin or a combination of DPCC and albumin [65]. BALF was found to be an excellent vehicle in which to suspend nanoparticles without altering their inflammatory or toxic potential [65]. Skebo et al. tried to reduce agglomeration by adding 0.1% sodium dodecyl sulfate (SDS) to silver nanoparticles [68]. To avoid direct toxic effects of SDS on cell viability, particles were washed twice with ultrapure water after adding 0.1% SDS and then introduced into cell cultures. Skebo et al. observed that the addition of SDS slightly decreased the agglomeration and hence increased the uptake of particles within cells [68].

While surface modifications allow the particles to be stabilized and avoids agglomeration, it also raises concern that they may shield or influence the effects of nanomaterials on biological systems [26, 69, 70]. The durability or stability of such surface coatings inside a biological environment is another critical issue that needs to be understood in order to unravel the toxicological consequences of nanoparticles. Quantum dot (QD) cores possess unique optical and electrical properties, but these cores are coated with different materials to make them biologically compatible/active [71]. However, Hoshino et al. reported that the exposure of QD surface coatings to the acidic and oxidative environments of endosomes may cause their decomposition and subsequent release into cytoplasm [72]. This can in turn expose the metalloid core, which may be toxic or pave the way for unforeseen reactions of the QD inside the cellular environment.

In vivo toxicity studies

In vivo tests are time-consuming, expensive, and involve ethical issues. In vitro toxicity tests, on the other hand, have been the first choice for most researchers working with nanomaterials. This can be attributed to the fact that these in vitro assays are faster, convenient, less expensive, and devoid of any ethical issues. However, the complex cell–cell and cell–matrix interactions, the diversity of cell types, and hormonal effects present in vivo are all missing from cultured cellular systems. Studying the long-term chronic effects of the test compound is also not possible without in vivo experiments.

There are studies that have suggested that in vitro screening studies do not reflect the actual effects of nanomaterials in their in vivo counterparts [28, 73]. Sayes et al. investigated the reliability of in vitro systems at predicting the in vivo pulmonary toxicity of fine ZnO particles and ZnO nanoparticles in rats, and concluded that in vitro cell culture systems do not precisely forecast the pulmonary hazards associated with in vivo exposure to ZnO particles [74].

Due to time demands, studies related to nanomaterials are shifting from in vitro to in vivo settings. Nanomaterial toxicologists have explored the effects of a variety of nanomaterials in animal experiments. However, in vivo studies with nanomaterials, unlike studies involving chemicals/compounds, are interlaced with many challenges (Fig. 3). The in vivo dose used for experiments should be derived from the quantity of nanoparticles exposed in the actual scenario. However, determining the quantity of nanoparticles in air, water, soil or any consumer product is a technical challenge due to their tiny size and the small quantity present. Even if the dose of nanoparticles is known, exceeding a certain dose in experiments is not advisable due to increased agglomeration of nanoparticles. The biodosimetry or biodistribution of nonfluorescent, nonradioactive, nonmagnetic nanoparticles is almost impossible.

When in vivo treatment is given for any test substance, it should be ensured that the vehicle is isotonic and nontoxic, and that the nanoparticle is well dispersed in the vehicle. Since nanoparticles are very susceptible to agglomeration owing to their increased relative surface area, they may not form a stable suspension in the physiological solutions suitable for in vivo exposure. The poor dispersion of nanoparticles during in vivo exposure negatively affects their biological distribution and subsequent activity [75]. Therefore, the results from such studies can be misleading and will differ from study to study. There are studies in the literature in which phosphate-buffered saline has been used as a vehicle for in vivo exposure, despite this being a poor dispersion agent [28, 65]. The dispersion medium itself should be fully characterized for its chemical properties and should not alter the biological activity of the test nanoparticle. Finding an appropriate vehicle for different routes of exposure during in vivo studies is still a challenge for toxicologists. Buford et al. and Sager et al. have reported the use of protein or lipid or a protein–lipid combination in the dispersion medium to get a stable nanoparticle suspension [65, 75]. Vehicles devoid of proteins or lipids produce larger agglomerates. BALF has been reported to be an effective dispersion medium for nanoparticles that does not mask the biological activity of the surface [65]. Buford et al. and Sager et al. have also reported that the addition of protein alone or DPPC (lipid) alone, in the same concentration as that of BALF, did not result in satisfactory dispersions [65, 75]. However, the addition of both protein and DPPC was efficient at significantly reducing agglomerate size. However, the use of BAL may give rise to problems related to reproducibility. Porter et al. have proposed that a synthetic dispersion medium which mimics lung fluid can be used as a vehicle for nanomaterial

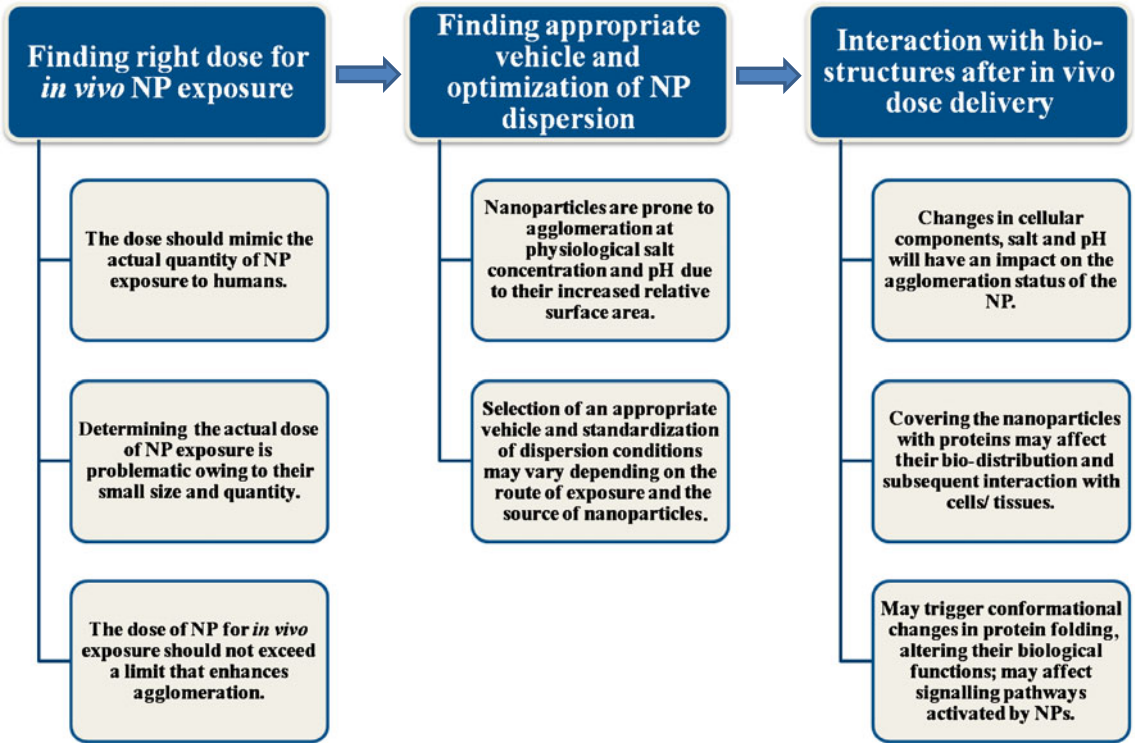

Fig. 3 Various steps and challenges/issues associated with in vivo nanotoxicity studies

toxicology studies, as it is biocompatible, inexpensive, and devoid of inter- and intralaboratory variations [76].

Even after selecting the most suitable dispersant and optimizing its dispersion conditions, a problem may arise in dispersing the same nanoparticles from different sources. Buford et al. highlighted this problem with CNTs when they observed variable dispersion characteristics of the CNTs from different sources in the same vehicle [75]. DLS may serve as a useful tool in such cases to determine the dispersion behavior/agglomeration status of the nanoparticle suspension in different conditions, and may thus help to find the best dispersion vehicle and method of dispersion. Searching for a dispersant and standardizing the dispersion conditions for a nanoparticle may not be apt for all routes of exposure, and will demand different optimization strategies depending on the route of exposure. For example, if the intratracheal delivery of nanoparticles occurs in BAL, then the intravenous delivery vehicle must be based on saline. Moreover, once inside the body, different salt concentrations and variable pH values may change the agglomeration status of the nanoparticle suspension.

When nanoparticles get inside the body, they come into contact with different biomolecules, especially protein. There are reports on the association of protein with nanoparticles and the formation of a "protein corona" [77, 78]. This could lead to altered properties of nanoparticles, thereby influencing their biodistribution and interactions with cells and biostructures. The binding of protein with nanoparticles may trigger conformational changes in protein folding, altering its biological function and affecting the signaling pathways activated by nanoparticles.

The importance of in vivo studies in nanomaterial toxicology and the challenges encountered in such studies have been discussed in detail by Fischer and Chan [21]. They have suggested that understanding the pharmacokinetics of the test nanoparticle should be the initial step in understanding its biological safety/toxicity. Pharmacokinetics is the study of the mechanisms of absorption, distribution and metabolism, and the effects and routes of excretion of the drug/compound or its metabolite. A thorough quantitative analysis of the pharmacokinetics of nanoparticles indicates the target tissues/cells, the residence time, and the time and dose required to manifest toxicity. This information can then be used to plan focused studies that involve only the target cell and help decipher the molecular basis of toxicity. This approach will also help to maximize the correspondence between in vivo and in vitro studies. A general conclusion about the pharmacokinetic behavior of nanomaterials cannot be drawn at present because of a lack of data and the fact that any difference in the physicochemical properties might change the pharmacokinetics [21]. However, before initiating pharmacokinetics studies, the route of nanoparticle exposure should be chosen carefully and should mimic the portal of entry for nanoparticles in the natural scenario.

Conclusion

Humans and other living organisms are exposed to nanomaterials, since they can be found in a wide array of products available in the consumer market. Nanoparticles are used in sunscreens and cosmetics due to their transparent appearance and enhanced efficacy. They are also used in tennis rackets and baseball bats to improve their strength and make them lighter. The textile industry is also using nanotechnology to produce stain-, wrinkle- and water-resistant clothing [79]. Silver and ZnO nanoparticles are used in food packaging as well as daily life appliances like washing machines and water purifiers [80].

The safety/toxicity aspects of nanomaterials have lagged far behind the rate at which they are being produced. This can be attributed to the lack of any guidelines and the absence of a consensus among researchers on experimental protocols or study designs in this field, as well as the unique properties of nanoscale materials, which cause problems during the toxicological assessment of novel nanomaterials. All of these factors give rise to conflicting and irreproducible results and slow down the growth of this field.

This review is an attempt to critically compile and analyze, from the available pool of information, different methods and challenges/issues associated with nanotoxicity studies. These include characterization, nanoparticle uptake, in vitro toxicity assays, agglomeration, in vivo study, and ecotoxicity. New experimental approaches, guidelines, and protocols are needed to determine the toxicity of nanomaterials. Until this is done, researchers should try to analyze the problems from as many aspects as possible; for instance, a multi-technique analytical approach should be employed for nanoparticle characterization. The fact that nanoparticle properties vary with the surrounding environment should be kept in mind. A multidisciplinary team effort involving material scientists, molecular biologists, toxicologists and physicists is required in nanotoxicology, as this will enable the different facets of nanotoxicology to be interlinked, thus aiding in our understanding of cellular responses to nanomaterial exposure and the mechanisms involved in them. The problems and issues faced during various in vitro and in vivo studies concerning nanomaterials should be openly reported and discussed in the literature. This will help to identify a solution as well as alert beginners beforehand, thus saving time and effort.

Finding answers to the present challenges and using new and upcoming technologies/systems/methods will not only help to elucidate the toxicities of various nanomaterials but will also be beneficial to nanotechnology, paving the way for safer products and a better quality of life.

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